Comparison of rapid cultural methods for the detection of Salmonella species

Margot, H ; Stephan, Roger ; O'Mahony, E ; Iversen, C

DOI: https://doi.org/10.1016/j.ijfoodmicro.2013.02.013

Comparison of rapid cultural methods for the detection of *Salmonella* species.

H. Margot[1], R. Stephan[1], E. O`Mahony[2], C. Iversen[2]

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, CH-8057 Zurich, Switzerland

[2]Quality and Safety Department, Nestlé Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne, Switzerland

* Corresponding author:

R. Stephan, Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstr. 272, CH-8057 Zurich, Switzerland, Phone 0041-44-6358651, fax 0041-44-6358908, e-mail stephanr@fsafety.uzh.ch

Abstract

Three newly developed rapid cultural methods (Rapid *Salmonella*, Precis™ *Salmonella,* IBISA *Salmonella*) for the detection of *Salmonella* spp. were compared to a reference method. All methods performed comparably on inclusivity/exclusivity testing. Similar limits of detection were observed for all methods with milk, cocoa and bouillon matrices. Some tea varieties appeared to disturb the normal color formation of all selective agars tested.

Keywords: rapid cultural methods, *Salmonella* detection, food matrix, inclusivity& exclusivity

1. Introduction

Traditional culture methods for the isolation and identification of *Salmonella* from food involve enrichment in a primary non-selective broth, such as lactose broth or buffered peptone water (BPW). This is followed by secondary selective enrichments in two media chosen from Rappaport-Vassiliadis (Soya) broth (RV(S)), selenite cystine broth and/or a tetrathionate broth. After plating on two selective agars, the suspect colonies are confirmed biochemically and serologically (ISO 6579:2002; BAM, 2011). The complete procedure requires at least three days to obtain a negative result and up to seven days for a confirmed positive result. Cultural methods have the advantage that they are capable of detecting viable cells, however the testing of large sample numbers is time consuming and labor intensive (Maciorowski et al., 2006). Production lots often need to be held in storage for several days during *Salmonella* testing. Therefore, the demand exists for faster yet still reliable and sensitive methods. The close relationship with other genera such as *Escherichia coli*, *Cronobacter* and *Citrobacter* spp. presents another challenge to the cultural detection of *Salmonella* spp.. These organisms compete in the enrichment media and/or can cause false positive reactions in *Salmonella* tests. Particularly in foods with a high initial background flora, low levels of *Salmonella* might be overgrown by the competing bacteria. (D`Aoust et al., 1992; Bennett et al., 1999) Newly developed cultural methods combine a single enrichment in a selective broth at an elevated temperature followed by isolation on a chromogenic agar. Background organisms are suppressed by the selective agents in the broth and agar and/or can be differentiated from *Salmonella* via the color reaction on the selective agar. Elimination of the non-selective enrichment step means the risk of overgrowth of *Salmonella* by competitors is reduced and that a negative result can be obtained in 44-48 hours.

2. Material and methods

2.1. Methods

The inclusivity/exclusivity and ability to recover *Salmonella* from difficult food matrices was evaluated for the following rapid cultural methods: Rapid *Salmonella* (Bio-Rad, Marnes-la-Coquette, France), Precis™ *Salmonella* (Oxoid, Pratteln, Switzerland) and IBISA *Salmonella* (AES chemunex, Bruz cedex, France). Rapid *Salmonella* and IBISA *Salmonella* include a supplement to BPW, which contains both nutrients to resuscitate injured *Salmonella* and inhibiting substances against competitor organisms. The Precis™ *Salmonella* method includes the ONE Broth-*Salmonella,* which is a complete selective enrichment medium. After the enrichment in the respective broths, aliquots are streaked on Rapid`*Salmonella* agar, Brilliance™ *Salmonella* agar and IBISA agar. *Salmonella* can be differentiated from non-target bacteria based on colony color and positive results are confirmed with a latex test. The reference method used was based on ISO 6579: 2002 using BPW, RVS, XLD and ChromID (bioMérieux, Marcy l`Etoile, France) for the detection of *Salmonella* species, this version of the standard method has been validated using ISO 16140 (MicroVal certificate number MV2007-LR06).

2.2 Strains

Forty-nine *Salmonella enterica* subsp. *enterica* strains and 32 non-*Salmonella* strains were used to determine inclusivity (sensitivity) and exclusivity (specificity) in the different media. The strains were chosen to provide a variety of serovars/species commonly found in food products and included strains that had given unexpected results in previous method evaluation studies. All strains were natural isolates obtained from our in-house collections.

2.3 Determination of doubling times

For the investigation of doubling times in the four enrichment broths (ONE broth-Salmonella, BPW+ Rapid`*Salmonella* supplement, BPW+ IBISA *Salmonella* supplement, BPW and RVS), overnight cultures of six *Salmonella* strains, as well as one strain each of *Cronobacter*

sakazakii, Shigella flexneri, Escherichia coli and Citrobacter freundii were grown in Tryptic Soy Broth (TSB, Oxoid) at 37 °C and serially diluted to 1: 10^{-6} in 9 ml of the recommended broth for each method. A 400 µL aliquot of each dilution from 1: 10^{-2} to 1: 10^{-6} was pipetted into duplicate wells of a Bioscreen C honeycomb plate. The plates were incubated in a Bioscreen C optical density reader (iLF bioserve e. K., Langenau, Germany) for 24 hours at 41.5 °C and the OD_{600nm} of the wells was measured every 15 minutes. The strains were similarly prepared in BPW and were incubated in the Bioscreen C for 24 hours at 37 °C. The doubling time for each strain was calculated using the regression equation for the linear trendline.

2.4. Limit of detection

For the determination of the limit of detection (LOD), four different food matrices (skimmed milk powder, sugar free cocoa, black tea and bouillon powder with Mediterranean herbs) were artificially inoculated. The LOD_p is the contamination level (cfu/g) leading to a positive result with a specified probability, p. The LOD_{50} and LOD_{95} were calculated for each method with four different food matrices, specifying the smallest quantity of *Salmonella* that can be detected with a probability of 50% or 95%, respectively. An overnight culture of *Salmonella* Tennessee strain S511 was diluted to achieve inoculation levels of approximately 3 CFU/25 g, 1 CFU/25 g and 0.3 CFU/25 g, with six replicates per inoculation level. Two 25 g portions of uninoculated sample served as negative controls and one positive control was inoculated with approximately 10 CFU/25 g.

For the skimmed milk and sugar free cocoa powder matrices, 25 g of each food product was enriched in 225 mL of the specific broth for each method. For the black tea and Mediterranean bouillon powder matrices, 25 g of the sample was added to 900 mL of broth. This higher dilution is routinely used in commercial testing laboratories as it has been found to be necessary to prevent growth inhibition of the target organism from the herb, spices and salt content of these matrices. For the rapid cultural methods, the enrichments were incubated for 16-20 hrs at 41.5 °C before streaking 10 µL onto selective medium. For the

121 reference method, primary enrichment was carried out in BPW, with 100 mL of a 10 %
122 solution of non-fat dry milk (NFDP) added to the BPW for enrichment of cocoa powder. All
123 BPW enrichments were incubated for 16-20 hrs at 37 °C, before transfer of 0.1 mL to 10 mL
124 of RVS broth, followed by incubation at 41.5 °C for 18-24 hrs. A 10 µL aliquot was then
125 streaked onto selective agar.
126

127 Based on the qualitative results statistical analysis was carried out by application of the
128 EXCEL sheet PODLOD.xls (Wilrich & Wilrich, 2009). The LOD_{50} with confidence limits for
129 each method with each matrix were calculated (table 3).
130
131
132
133
134
135
136
137
138
139
140
141

142 ## 3. Results & Discussion

143 ### 3.1. Inclusivity/ exclusivity

144 All methods performed with 100% specificity and sensitivity. Where growth of non-
145 *Salmonella* strains occurred, these could be differentiated from target *Salmonella* strains by
146 the colony colour (see table 1a &b). On the IBISA agar, some *Salmonella* serovars only
147 showed weak growth. Non-target organisms which showed growth similar to *Salmonella* in
148 the selective broths (*Cronobacter malonaticus* and *Cronobacter muytjensii*) did not grow on
149 the chromogenic agar plates of the three alternative methods.

150

151 ### 3.2. Growth rates in the selective broths

152 The growth rate of *Salmonella* in BPW, RVS and in broths from the Precis *Salmonella* and
153 IBISA *Salmonella* methods were found to be in a similar range (table 2). For all rapid method
154 broths the growth of *E. coli* and *S. flexneri* was inhibited. The doubling time of *C. sakazakii*
155 ranged from 18.62 minutes in BPW to 44.94 minutes in Rapid *Salmonella* selective broth.
156 RVS and Rapid *Salmonella* inhibited the growth of the *C. freundii* strain. Doubling times for
157 this strain in the other broths ranged from 25.04 to 41.15 minutes. *Cronobacter* and
158 *Citrobacter* are the main background organisms that pose problems for the isolation of
159 *Salmonella*. These organisms can generally grow on *Salmonella* selective agar, but do not
160 produce typical *Salmonella* colony morphologies. Therefore false positive colonies should not
161 be a cause for concern. However, the potential exists for false negative results due to
162 overgrowth of the target organisms from the competing flora.

163

164 ### 3.3 Detection limit in the food matrix

165 For the milk powder, cocoa powder and Mediterranean bouillon powder, all methods could
166 detect levels of contamination ranging from < 0.008- 0.144 CFU/g of product. For black tea

the LOD_{50} ranged from 0.051 to 0.219 CFU/g. When black tea was enriched with the different broths and isolated on the selective agars, atypical colouration of the colonies made it difficult to interpret the result on the plates (table 3).

Further analysis was performed with different tea varieties by overnight enrichment of 25 g in 900 mL BPW inoculated with approximately 10 CFU of *Salmonella* Congo S617. The enrichment was then streaked on 6 different selective agars for presence/absence testing. The selective agars were: XLD, BGA, chromID™ *Salmonella* (bioMérieux), Rapid Salmonella (BioRad), Precis™ *Salmonella* (Oxoid) and IBISA *Salmonella* (AES chemunex). Two tea varieties appear to contain ingredients, which disturb the normal color formation of all selective agars tested.

In conclusion, newly developed rapid cultural methods present a time saving of 48 hours for the cultural detection of *Salmonella* spp. in food samples. This saving is achieved by omitting the non-selective enrichment step and using a fast latex agglutination test for confirmation of presumptive positive colonies. The tested methods were as sensitive and specific as the reference method when pure cultures of target and non-target strains were tested. Difficult matrices such as black tea can pose a problem with false negative results due to atypical colony colors.

References:

Bacteriological Analytical Manual. Chapter 5 Salmonella November 2011 Version US FDA. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070149.htm

Bennet, A. R., MacPhee, S., Betts, R., Post, D., 1999. Use of pyrrolidonyl peptidase to distinguish *Citrobacter* from Salmonella. Letters in Applied Microbiology 28,175-178.

ISO International Standard 6579, Microbiology of food and animal feeding stuffs-Horizontal method for the detection of *Salmonella* spp. 4 th edition, 2002-07-15.

D`Aoust, J. Y., Sewell, A. M., Warburton, D. W., 1992. A comparison of standard cultural methods for the detection of foodborne *Salmonella.* International Journal of Food Microbiology **16,** 41-50.

Maciorowski, K. G, Herrera, P., Jones, T., Pillai, S. D, Ricke, S. C**.,** 2006. Cultural and immunological detection methods for Salmonella spp. in animal feeds- a review. Veterinary Research Communications **30,**127-137.

Wilrich, C., Wilrich, P., 2009. Estimation of the POD Function and the LOD of a Qualitative Microbiological Measurement Method. Journal of AOAC International 92,1763-1772.

212

213 **Table 1:** Inclusivity results for each method evaluated.

Salmonella strains	N	Rapid *Salmonella* (Magenta*)	Precis *Salmonella* (Purple*)	IBISA *Salmonella* (Green*)	Reference method XLD (Black*)	Reference method ChromID (Pale pink/mauve*)
Salmonella Agona	1	+	+	+	+	+
Salmonella Albany	1	+	+	+	+	+
Salmonella Anatum	3	+	+	+	+	+
Salmonella Ank	1	+	+	+	+	+
Salmonella Bergen	1	+	+	+	+	+
Salmonella Bracknell	1	+	+	+	(clear)	+
Salmonella Cerro	1	+	+	+	+	+
Salmonella Congo	1	+	+	+	+	+
Salmonella Chittagong	1	+	+	+	+	+
Salmonella Denver	1	+	+	+	+	+
Salmonella Derby	1	+	+	+	+	+
Salmonella Enteritidis	2	+	+	+	+	+
Salmonella Ekotedo	1	+	+	+	(clear)	+
Salmonella Farmsen	1	+	+	+	+	+
Salmonella Gambe	1	+	+	+	(clear)	+
Salmonella Ibandan	1	+	+	+	+	+
Salmonella Infantis	2	+	+	+	+	+
Salmonella Kentucky	1	+	+	+	(clear)	+
Salmonella Kibi	1	+	+	+	+	+
Salmonella Kinondoni	1	+	+	+	+	+
Salmonella Limete	1	+	+	+	+	+
Salmonella Maritzburg	1	+	+	+	+	+
Salmonella Mbandanka	1	+	+	+ (weak)	+	+
Salmonella Napoli	2	+	+	+	+	+
Salmonella Nashua	1	+	+	+ (weak)	(clear)	+
Salmonella Oranienburg	1	+	+	+	(clear)	+
Salmonella Panama	1	+	+	+	+	+
Salmonella Plymouth	1	+	+	+	+	+
Salmonella Rissen	1	+	+	+	+	+
Salmonella Salamae	1	+	+	+ (weak)	(clear)	+
Salmonella Sundsvall	1	+	+	+	+	+
Salmonella Tennessee	2	+	+	+ (weak)	+	+
Salmonella Thompson	1	+	+	+	+	+
Salmonella Typhimurium	5	+	+	+ (weak)	(clear)	+
Salmonella Uno	1	+	+	+	+	+
Salmonella Virchow	1	+	+	+	+	+
Salmonella Wagadugu	1	+	+	+	+	+
Salmonella Wagama	1	+	+	+	+	+
Salmonella Wagenia	1	+	+	+	(clear)	+
Inclusivity %		100%	100%	100%		100%

214

215 Footnotes: -, no growth, +, typical growth, + (weak), weak growth, N, number of strains tested

216 * expected typical colony color for *Salmonella* spp.

217

218

219

Table 1b: Exclusivity results for each method evaluated. * expected typical colony color for *Salmonella* spp.

Organism	Rapid *Salmonella* (Magenta*)	Precis *Salmonella* (Purple*)	IBISA *Salmonella* (Green*)	Reference method XLD (Black*)	Reference method ChromID (Pale pink/mauve*)
Enterobacter hormaechei	green	green	-	Atypical (yellow)	Atypical (turquoise)
Shigella flexneri	-	-	-	-	Atypical (white)
Enterobacter asburiae	-	green	-	-	Atypical (turquoise)
Proteus mirabilis	-	-	-	-	Atypical (beige)
Providencia alcalifaciens	-	beige	-	-	-
Cronobacter turicensis	dark blue	-	Purple	Atypical (yellow)	Atypical (dark blue)
Hafnia alvei	-	-	-	Atypical (clear)	Atypical (turquoise)
Klebsiella oxytoca	-	green	-	Atypical (yellow)	Atypical (blue green)
Cronobacter sakazakii	-	-	black	-	Atypical (green)
Enterobacter cloacae	-	-	-	-	-
Cronobacter malonaticus	-	-	-	Atypical (beige)	Atypical (blue)
Cronobacter muytjensii	-	-	-	Atypical (yellow)	Atypical (blue purple)
Escherichia coli	-	-	-	Atypical (yellow)	Atypical (turquoise)
Escherichia coli	-	-	-	Atypical (clear)	Atypical (turquoise)
Serratia liquefaciens	-	-	-	-	-
Enterobacter amnigenus	-	-	-	-	Atypical (turquoise)
Cronobacter sakazakii	-	-	-	Atypical (white)	Atypical (dark blue)
Enterobacter asburiae	-	green	-	Atypical (yellow)	Atypical (turquoise)
Serratia plymuthica	-	-	-	-	-
Citrobacter freundii	-	beige	-	Atypical (yellow)	Atypical (turquoise)
Citrobacter freundii	rosé	beige	-	Atypical (yellow)	Atypical (turquoise)
Citrobacter freundii	-	beige	-	Atypical (clear)	Atypical (turquoise)
Citrobacter freundii	-	-	-	-	Atypical (turquoise)
Enterococcus faecalis	-	-	-	-	-
Micrococcus luteus	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-
Listeria innocua	-	-	-	-	-
Pantoea spp.	-	-	-	-	-
Exclusivity %	100%	100%	100%	100%	

Footnotes: -, no growth,

* expected typical colony color for *Salmonella* spp.

220

221

Table 2: Doubling time (minutes) in base broths for each isolation method.

Organism	Rapid *Salmonella*	Precis *Salmonella*	IBISA *Salmonella*	BPW	RVS broth
Salmonella (average +/- STDEV)	35.3 +/- 16.4	27.3 +/- 8.5	28.4 +/- 8.8	21.9 +/- 1.9	29.7 +/- 6.3
Cronobacter sakazakii	44.94	26.94	21.49	18.62	43.70
Shigella flexneri	-	-	-	24.43	-
Escherichia coli	-	-	-	15.66	-
Citrobacter freundii	-	36.20	41.15	25.04	-

Footnote: -, no growth

250

251

252 **Table 3:** Limit of detection (CFU/g) with upper and lower 95 % confidence limits

	Food sample							
	Milk powder		**Bouillon powder**		**Black tea**		**Cocoa powder**	
Method	***LOD*$_{50}$**	**Confidence Limit**	***LOD*$_{50}$**	**Confidence Limit**	***LOD*$_{50}$**	**Confidence Limit**	***LOD*$_{50}$**	**Confidence Limit**
Reference	0.039	0.018-0.084	0.009	0.004-0.020	0.110	0.054-0.224	0.057	0.027-0.120
Rapid *Salmonella*	0.144	0.070-0.296	0.039	0.017-0.089	0.062	0.030-0.129	0.082	0.040-0.168
Precis *Salmonella*	0.057	0.027-0.119	0.036	0.016-0.079	0.051	0.024-0.107	0.088	0.043-0.181
IBISA	0.024	0.011-0.056	0.007	0.003-0.015	0.219	0.100-0.477	0.068	0.033-0.142

253

254